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CATEGORIA III

INFORME TRIMESTRAL DEL PROYECTO DE CONSERVACIÓN EX SITU DE ESPECIES DE INTERÉS DE ANFIBIOS

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INFORME TRIMESTRAL DEL PROYECTO

“LA CONSERVACIÓN EX SITU DE RANAS DEL ÁREA DE DONOSO, RENOVACIÓN POR TRES AÑOS”

Período: Mayo-Julio 2022

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Introducción

El 1 de septiembre de 2019, Minera Panamá S.A. (MPSA) y el Instituto Smithsonian de Investigaciones Tropicales (STRI) firmaron un acuerdo con el fin de extender el proyecto de conservación ex situ de anuros del área de Donoso por tres años. Con este acuerdo se continuarán los esfuerzos de conservación e investigación que contribuirán al manejo y preservación de los anfibios de Donoso, enfocándose en las especies de interés (EdI). Este proyecto es coordinado por el Proyecto de Rescate y Conservación de Anfibios de Panamá (PARC), que lleva a cabo las actividades de conservación ex situ dentro de instalaciones del STRI, ubicadas en Gamboa, provincia de Colón. En este informe se presentan los avances, de acuerdo a los objetivos acordados de este proyecto, realizados durante el período Mayo-Julio 2022.

Durante este trimestre, nosotros continuamos brindándole todo el cuidado requerido a las poblaciones de anfibios que mantenemos en el programa de conservación ex situ en Gamboa; al mismo tiempo, evitando o minimizando el riesgo de contagio del COVID-19 entre nuestros colaboradores. Estas actividades se realizaron siguiendo las indicaciones de las entidades de gobierno correspondientes y las medidas sanitarias sugeridas por el STRI. No obstante, las actividades que no están directamente vinculadas al cuidado y reproducción de los animales fueron limitadas, como aquellas relacionadas con trabajo de campo. Estas actividades se tendrán que planificar y reprogramar en el próximo trimestre.

Objetivo 1. – Mantener la instalación para la cría y reproducción de poblaciones en cautividad de las especies de anfibios de interés de Donoso.

Con relación a nuestra inversión en la capacitación de personal especializado en cría de anfibios, hemos realizado actividades. Tres estudiantes universitarios han continuado con sus investigaciones sobre técnicas de reproducción asistida en nuestras instalaciones para completar sus trabajos de graduación. La pasante Igli Arcia con estudios en medicina veterinaria, continuó participando y apoyándonos en la aplicación de técnicas de reproducción asistida y criopreservación de esperma de las ranas. A finales de julio, Igli pasó a ser colaboradora del proyecto, como Técnica de Investigación I. Además, recibimos el apoyo de dos voluntarios en este trimestre.

Realizamos trabajos de mantenimiento y reparación de equipo en la instalación de Gamboa, relacionados al mantenimiento periódico del generador eléctrico y de la planta de tratamiento de aguas residuales. Se repararon y ajustaron las bombas de agua de algunos contenedores.

Se reemplazaron tubos de luces blancas y todos los filtros de agua en los contenedores. Además, se cambiaron todas las lámparas de los tanques con ranas en el contenedor No.1 por lámparas con armazón plástico y luz UVB, las cuales no se corroen y se espera que tengan una vida útil más prolongada. El 15 de junio completamos el desalojo de los salones en Santa Cruz, donde temporalmente producíamos invertebrados, y nos mudamos a la nueva edificación de 150 m².

La producción de invertebrados ha continuado ininterrumpidamente, aún durante la mudanza. Nos tomó un par de semanas reorganizarnos en el nuevo edificio, pero sin disminuir nuestra producción usual de invertebrados. Continuamos produciendo grillos, dos especies de moscas de las frutas, colémbolos, larvas de polilla de despena y polillas de cera ("waxworms"), lombrices de tierra, una especie de cucaracha, larvas de dos especies de escarabajos ("mealworms" y "superworms") y cochinillas para alimentar a las poblaciones de anfibios en cautiverio. En estos meses, la producción de invertebrados sobrepasó el nivel requerido para alimentar a las ranas que mantenemos en Gamboa, lo cual es conveniente en caso de que ocurra alguna mortalidad inesperada de invertebrados y, aun así, poder cubrir las necesidades de alimentación de las ranas.



El edificio nuevo para la producción de invertebrados en Gamboa cuenta con dos salones para la producción de invertebrados: (1) donde se mantiene una temperatura de 21-22°C para la cría de moscas de las frutas, colémbolos y cochinillas (imagen izquierda), y (2) donde se tiene una temperatura de 27-29°C para la producción de grillos, cucarachas, larvas de escarabajos y polillas (imagen derecha). La diferencia en temperaturas entre ambos salones es necesaria para la reproducción óptima de las varias especies de invertebrados que producimos. Además, se cuenta con un depósito para almacenar los insumos que son requeridos para producir los invertebrados. El edificio tiene un área total de ~150 m².

Los números de individuos de las poblaciones en cautiverio de las especies de anfibios del área de Donoso, que manteníamos al 31 de julio de 2022, en Gamboa eran:

Especie	Machos	Hembras	Juveniles	Total	Reproducción en cautiverio	Renacuajos
<i>Andinobates geminisae</i>	40	70	190	300	+	25
<i>Atelopus varius</i>	199	170	312	681	+	~1200
<i>Craugastor evanesco</i>	13	17	0	30	-	-
<i>Gastrotheca cornuta</i>	6	1	0	7	-	-
<i>Oophaga vicentei</i>	34	35	66	135	+	~60
Total	292	293	568	1153		~1285

Nota: *Craugastor evanesco* y *Gastrotheca cornuta* no presentan etapa de larvaria o renacuajo libre.

Al 31 de julio de 2022, teníamos 1153 ranas de cinco especies de anuros del área de Donoso. En el cuadro no se incluyen a los individuos de *Atelopus varius* que se utilizaron para el ensayo de liberación y los experimentos con toxinas. Entre las especies, que se mantienen en cautiverio, están cinco Especies de Interés (EdI) del Proyecto Mina de Cobre Panamá: *Andinobates geminisae*, *Atelopus varius*, *Craugastor evanesco*, *Gastrotheca cornuta* y *Oophaga vicentei*.

Para las especies *Andinobates geminisae*, *Craugastor evanesco* y *Oophaga vicentei* han sido colectados suficientes individuos fundadores con el fin de iniciar la población ex situ. Sin embargo, aún es recomendable obtener más individuos de *Atelopus varius* (6-10 hembras de la población en Nuevo Petaquilla) y *Gastrotheca cornuta* (14 machos y 19 hembras) para obtener una representatividad adecuada de su diversidad genética. La especie *G. cornuta* sido particularmente difícil de encontrar; por lo que, nos estaremos concentrando en la búsqueda de más individuos de esta especie. Esperamos reanudar expediciones en este año, con el fin de recolectar más fundadores de *G. cornuta*.



Post-metamorfo resultado de la fecundación *in vitro* con espermatozoides y oocitos frescos obtenidos de una pareja estimulada hormonalmente, como parte del trabajo colaborativo de la Dra. Gina Della Togni, quien es especialista en técnicas de reproducción asistida.

Durante este período, se mantuvieron 46 parejas de *Andinobates geminisae* en los tanques de reproducción, incluyendo parejas constituidas por individuos de la segunda generación filial (F2) nacidos en cautiverio. Entre estas parejas hubo varios eventos de reproducción. Resultado de estos eventos reproductivos, para el final del período, se tenían 25 renacuajos y 190 juveniles. Se observó una disminución en la puesta de huevos y renacuajos de *A. geminisae*, lo cual ha sucedido en otras ocasiones durante esta época del año. Los juveniles de esta especie fueron desparasitados. Además, mantuvimos 35

parejas de *Oophaga vicentei* en tanques de reproducción durante este período. Encontramos unos 60 renacuajos de *O. vicentei*, algunos en los receptáculos de las plantas bromelias artificiales, al final de este período. Continuamos con el tratamiento tópico de las hembras reproductoras de *O. vicentei* con gotas de gluconato de calcio y el uso de agua reconstituida filtrada por ósmosis inversa para criar a los renacuajos, con el propósito de reducir la aparición del síndrome de patas débiles (SLS) en los post-metamorfos. Síndrome que no les permite moverse con normalidad para alimentarse y, como consecuencia, mueren. En este período, no hubo post-metamorfos de *Oophaga vicentei* que presentasen el SLS. Se obtuvieron unos 66 juveniles sanos de *O. vicentei*, incluyendo juveniles que pertenecen a la segunda generación filial (F2) nacidos en cautiverio.

Se mantuvieron 13 parejas de adultos de *Craugastor evanescens* en tanques para su reproducción. No se realizaron los ensayos con tratamientos hormonales en esta especie durante el trimestre. Adicionalmente, los cuatro individuos provenientes del río Ubero de Donoso aún se mantienen aislados. Estos individuos no se contabilizaron en el cuadro anterior, ya que surgieron dudas sobre su identificación; por lo que, estaremos secuenciando el ADN mitocondrial de un par de genes para determinar la especie de estos individuos.

En el trimestre Mayo-Julio, finalizó la salida de post-metamorfos de los tanques con renacuajos de una masa de huevos de *Atelopus varius* que fue puesta en enero. Adicionalmente, de la fecundación *in vitro* de los oocitos de dos hembras utilizando orina espermática fresca, mencionada en el trimestre pasado, se obtuvo sólo un post-metamorfo. Todavía, se mantienen tanques con renacuajos de cuatro parejas, dos de éstas habían sido estimuladas hormonalmente, a partir de las masas de huevos depositadas en el trimestre anterior. De los tanques de tres de estas puestas, ya empezaron a salir post-metamorfos. En este período, se colocaron tres parejas en tanques de reproducción; sin embargo, solamente una pareja depositó huevos, de los cuales tenemos renacuajos. Al final del período, manteníamos un total aproximado de 1200 renacuajos de *A. varius*.

Como parte de los preparativos para un experimento en el laboratorio y un ensayo de liberación con *Atelopus varius*, continuamos realizando cruces de individuos cuyo linaje proviene de poblaciones de tierras bajas (i.e., Donoso) con individuos de un linaje proveniente de poblaciones de tierras altas. El número de individuos resultantes de los cruces para este experimento no se incluyen en el cuadro antes presentado. Durante Mayo-Julio, se colocó una pareja en tanques de reproducción; pero, el macho no abrazó a la hembra y, por ende, no se reprodujeron. No obstante, aún se mantiene un tanque con renacuajos de una puesta de huevos que fue depositada en enero y post-metamorfos provenientes de ésta. Con este experimento y ensayo de liberación determinaremos si una mayor diversidad genética de los individuos confiere una mayor resistencia o tolerancia al hongo patógeno de anfibios, *Batrachochytrium dendrobatidis*.

Todas las ranas de *Gastrotheca cornuta* se mantuvieron individualmente en tanques y fueron alimentadas de manera asistida. Cuando la única hembra que tenemos esté “grávida” nuevamente, la volveremos a emparejar. A través de estos intentos y eventos reproductivos, seguimos conformando paulatinamente poblaciones estables en cautiverio de las especies EdI de Donoso. En este sentido, para las especies que se han reproducido con mayor frecuencia, *Atelopus varius*, *Andinobates geminisae* y *Oophaga vicentei*, tenemos individuos fundadores (parentales) y descendientes de la primera generación filial (F1) y la segunda generación filial (F2) en las poblaciones de éstas que están en cautiverio. Es más, hemos producido un excedente de individuos de *A. varius*, los cuales hemos destinado a ensayos de liberación y experimentos

sobre toxinas. En el caso de *Craugastor evanescens* y *Gastrotheca cornuta*, solamente se tienen parentales y descendientes F1.

En este trimestre, analizamos 177 muestras de piel ranas con hisopos, obtenidos a partir de una rana por cada tanque, para asegurarnos de que el hongo quitridio patógeno de anfibios no se encuentre dentro de nuestra instalación en Gamboa. Usando la técnica molecular de qPCR, determinamos que todas estas muestras eran negativas, por lo que este hongo patógeno no está presente en nuestra colección de ranas vivas.

Adicionalmente, se ha continuado con la introducción de datos de los individuos de Donoso en la base de datos “Zoological Information Management System” (ZIMS) (<http://www2.isis.org>), lo cual facilita el manejo del inventario de animales, el control de la conformación genética y demográfica de las colecciones de animales vivos, la identificación de animales no relacionados para realizar cruces, el mantenimiento de un registro del estado de salud y el tratamiento que se le ha dado a cada individuo, entre otros.



Pareja de *Atelopus varius* dentro de un tanque de reproducción (imagen izquierda), nótese el abdomen distendido de la hembra (de espalda) debido a que contiene un gran número de oocitos, por lo que se le dice que está “grávida”. Post-metamorfos de *A. varius* recién salidos del agua de uno de los tanques de reproducción (imagen derecha).

Objetivo 2. – Continuar el trabajo para ensayos de liberación de anfibios en el área de Donoso.

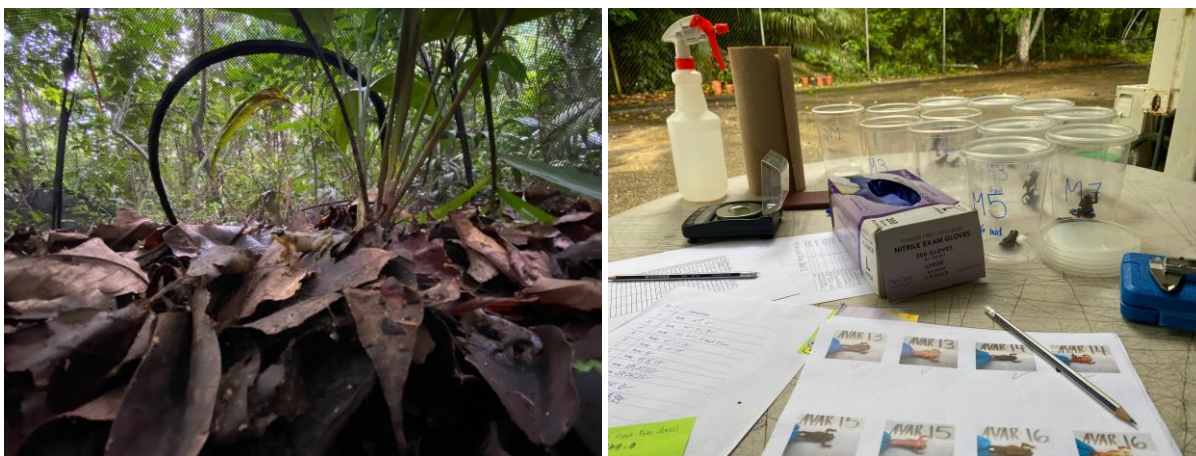
En este trimestre, se llevó a cabo el proyecto piloto para probar el tipo de mesocosmo (encierro) que será utilizado en liberaciones suaves (“soft releases”) y determinar cuál es la densidad de individuos más adecuada para los ensayos de liberación de ranas *Atelopus varius*. En este ensayo probamos un tipo de mesocosmo para contener a las ranas durante liberaciones suaves, que según un ensayo previo resultó cualitativamente ser el mejor (i.e., Purrfect Pen). Durante tres semanas, se mantuvieron individuos de *A. varius* a tres densidades (i.e., 1, 3 y 6 individuos) con tres réplicas por cada densidad. Para este ensayo, utilizamos 30 individuos que inicialmente se mantenían a 24-25°C y que fueron aclimatados a 26-27°C por un mes, dentro de uno de los contenedores de nuestra instalación, con el propósito de acostumarlos a las temperaturas del área. El ensayo se realizó en un área boscosa adyacente a la instalación del PARC en Gamboa y tuvo una duración de tres semanas. Los mesocosmos eran inspeccionados con frecuencia desde su parte exterior para verificar su integridad y que tuviesen frutas para atraer insectos. Semanalmente, el interior de todos los mesocosmos era revisado y los individuos encontrados eran pesados y medidos para determinar su condición corporal. El análisis de los hisopados de la piel de los individuos indica que éstos no fueron infectados por el hongo patógeno de anfibios, durante el ensayo. En el próximo trimestre, analizaremos los datos de supervivencia y cambios en la condición corporal según la densidad de individuos.

Varias especies de anfibios poseen toxinas en la piel, las cuales les brindan cierta protección contra los depredadores. Las ranas dendrobátidas, *Andinobates geminisae* y *Oophaga vicentei*, y las ranas arlequines, *Atelopus varius*, son especies que en condiciones silvestres presentan toxinas en su piel. Las toxinas de la piel de estas ranas están constituidas por alcaloides. Los alcaloides de las especies de las ranas dendrobátidas y las ranas arlequines pertenecen a grupos químicos distintos, por lo que tienen propiedades diferentes. Se conoce que la fuente de los alcaloides presentes en las ranas dendrobátidas provienen de las presas que forman parte de su dieta natural; mientras que, no se ha establecido claramente cuál es el origen de los alcaloides en las ranas arlequines, se piensa que pueden ser sintetizados parcialmente, adquiridos a través de la dieta o provistos por organismos simbiotes (e.g., bacterias) de su piel. No obstante, debido a que los individuos criados en cautiverio carecen de toxinas, hemos iniciado experimentos con el fin de determinar si existe la posibilidad de restablecer artificialmente las toxinas de la piel en ranas criadas en cautividad. En colaboración con el Dr. Ralph Saporito, profesor de John Carroll University, y Dr. Luke Linhoff, becario postdoctoral del Instituto Smithsonian, se ha estado llevando a cabo un experimento en el cual se alimentan a 10 parejas de *O. vicentei* y 10 parejas de *A. geminisae* con insectos espolvoreados con un alcaloide liposoluble, tres veces por semana. Adicionalmente, en colaboración con el Dr. Phillip Jervis y Dr. Gonçalo Rosa, asociados al Imperial College London y al Institute of Zoology, hemos iniciado un experimento con 30 individuos de *A. varius*, algunos de los cuales han sido alimentados con insectos inyectados con un alcaloide guanidinio. Estos experimentos cuentan con la aprobación de su protocolo por parte el IACUC (i.e., Comité Institucional para el Cuidado y Uso de Animales) del STRI. El restablecimiento de las toxinas de la piel de estas ranas es crucial para disminuir o evitar su depredación, luego de ser liberadas.

Se completaron los análisis de los hisopados de piel del experimento de infección, relacionados a las investigaciones sobre las secreciones de la piel (“mucosoma”) de los anfibios. Nuestro colaborador Dr. Luke Linhoff estuvo varias semanas del mes de mayo en Panamá con el fin de continuar y completar los ensayos *in vitro* de las secreciones de la piel de las ranas.

Adicionalmente, durante este trimestre, él también ha estado analizando los datos y escribiendo sobre los resultados a su regreso a los EE.UU. Como se indicó en informes trimestrales anteriores, las conclusiones de estas investigaciones sobre las secreciones de la piel de los anfibios pueden tener implicaciones importantes para la liberación exitosa de ranas provenientes de poblaciones en cautiverio.

Adicionalmente, en este trimestre, publicamos un artículo en la revista científica *Biological Conservation* (Anexo 1), la cual es revisada por pares. En esta publicación, se incluyó una parte de los resultados obtenidos durante el ensayo de liberación de individuos de *Atelopus varius*, que realizamos en el año 2018 dentro del área de la concesión minera en Donoso. Específicamente, el artículo se refiere a los cambios en la comunidad de microorganismos de la piel en las ranas nacidas en cautiverio y que se mantuvieron dentro de mesocosmos, usados para la liberación suave de individuos. A partir de este trabajo, podemos concluir que la comunidad de microorganismos de su piel se restablece naturalmente en unos pocos meses después que los individuos son liberados, tornándose más parecida a la comunidad de microorganismos en la piel de individuos silvestres y con un aumento en las bacterias de la piel que potencialmente pueden producir sustancias antimicóticas.



Individuo de *Atelopus varius* dentro de uno de los mesocosmos (en el centro de la imagen izquierda). Identificación y toma de medidas y peso de los individuos que se encontraban dentro de los mesocosmos (imagen derecha), lo cual se realizó semanalmente.

Objetivo 3. – Realizar expediciones para recolectar ciertas especies de anfibios en riesgo en el área de Donoso.

Durante este período nosotros no realizamos expediciones para recolectar individuos. Como se indicó en informes anteriores, *Gastrotheca cornuta* es una Especie de Interés (EdI), de la cual se requieren más individuos fundadores para establecer adecuadamente una población ex situ en Gamboa. Esperamos realizar próximamente una expedición al sitio 4CS6 o a sitios cercanos a la costa.

Objetivo 4. - Estudio de los hongos patógenos de anfibios en la región de Donoso.

Debido a que no realizamos expediciones a Donoso en este trimestre, no obtuvimos muestras de piel para el análisis del hongo patógeno de anfibios, *Batrachochytrium dendrobatidis*.

Anexo 1. Artículo publicado: "Kueneman, J. G., M. C. Bletz, M. Becker, B. Gratwicke, O. A. Garcés, A. Hertz, W. M. Holden, R. Ibáñez, A. Loudon, V. McKenzie, L. Parfrey, B. Sheafor, L. A. Rollins-Smith, C. Richards-Zawacki, J. Voyles y D. C. Woodhams. 2022. Effects of captivity and rewilding on amphibian skin microbiomes. *Biological Conservation* 271:e109576." <https://doi.org/10.1016/j.biocon.2022.109576>



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Effects of captivity and rewilding on amphibian skin microbiomes

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ABSTRACT

Captive breeding to safeguard against extirpation in the wild is a practice for many animal groups. Animals in captivity experience reduced contact with natural substrates and other animals, and consume atypical diets that may alter naturally occurring microbial associations. Amphibian skin microbiomes are vital for amphibian health, protecting them from pathogens and aiding in development, immune system training, and fecundity. Thus, understanding how changes associated with captivity influence microbial communities and the health of captive-reared amphibians is an important consideration in captive breeding and reintroduction programs. Overarching patterns of amphibian microbial diversity in captivity have not been previously explored. Therefore, we conducted a meta-analysis of skin microbes from captive-managed and wild individuals of 18 salamander and frog species from temperate and tropical biomes. We found that microbial composition of captive and wild amphibians differed for all species. However, while the overall captivity effect on amphibian skin richness was significant, the direction of the captivity effect on diversity metrics and antifungal function differed depending on the host species. One species exhibiting a large skin microbiome shift in captivity is the variable harlequin frog, *Atelopus varius*. A soft-release of *A. varius* to outdoor mesocosms “restored” the microbiome through time, and frogs also increased antifungal function of their skin microbiome with time in mesocosms. Rewilding the microbiome may influence resistance to diseases such as chytridiomycosis. Indeed, evaluating the outcome of individual species is necessary until we have a cohesive approach to mediate shifts of amphibian skin microbes that result from captivity.

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1. Introduction

The number of species listed as vulnerable, endangered, or critically endangered has increased dramatically in recent decades (González-del-Pliego et al., 2019), expanding the implementation of a wide range of conservation responses and intervention strategies. In some cases, the threats to species survival are sufficiently imminent and severe that animals are rapidly moved into ex situ captive programs to stave off extinction (Gratwicke et al., 2016). Commonly, captive programs are established with an initial goal of reintroducing the species back into their native range once the threat has abated (Lyles and May, 1987; Griffiths and Pavajeau, 2008). However, to date the outcomes of reintroduction efforts have been mixed, with some notable failures to establish viable populations in the wild (e.g., Seddon et al., 2007; Robert et al., 2015). Conservation biologists have attempted to use diverse approaches to increase the likelihood of success in reintroduction efforts, including strategies to mitigate or dampen the detrimental effects of captivity (van Wieren, 2012). While this has traditionally meant incorporating pre-release conditioning or a soft-release of captive animals (Seddon et al., 2007), the role of the microbiome of captive animals, and how it differs from that of their wild counterparts, has not figured prominently in reintroduction biology until relatively recently (Redford et al., 2012).

The conventional wisdom for captive breeding is that the microbiome of captive animals is depleted or disrupted relative to their wild counterparts (Ross et al., 2019). The disruption of the microbiome in captivity may be due to use of artificial substrates, frequent cleaning of housing spaces, provision of a non-native diet, decreased habitat complexity and stabilized climatic conditions, and/or reduced species interactions and behaviors which are constrained in captivity (McKenzie et al., 2017). A depleted microbiome can have a wide range of detrimental health effects (Redford et al., 2012). For example, conditions in captivity may prevent colonization by beneficial microbiota that could contribute to disease defenses, proper absorption of nutrients, and other biological functions that are critical for health (Sommer and Bäckhed, 2013; Cénit et al., 2014). Indeed, this paradigm is supported by data from diverse animals in captive populations (Kong et al., 2014; Alfano et al., 2015; Cheng et al., 2015). The studies included an in-depth analysis of mammalian gut microbiota in captivity compared to wild mammals showing consistent compositional differences between wild and captive counterparts but inconsistent responses of microbial richness, including decreased diversity, no change, or increased diversity (McKenzie et al., 2017).

One compelling example of threatened species that could benefit from investigations on the microbiome is that of amphibians (Bletz, 2013; Rebollar et al., 2016; Walke and Belden, 2016). The Conservation Needs Assessment, completed in 2019, recommended 577 amphibian species for ex situ captive breeding programs (www.conservationneeds.org). Currently, 180 at-risk species are subsisting in captive breeding programs, with a goal to reintroduce healthy individuals to depleted populations in the wild. For many of these amphibian species, emerging pathogens, including ranaviruses and *Batrachochytrium* fungi, such as, *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal), are severe and persistent threats, affecting hundreds of amphibian species globally (Spitzen-van der Sluijs et al., 2016; Scheele et al., 2019). As the risk of disease emergence and spread continues to threaten amphibian populations, the reliance on captive breeding programs will likely expand, potentially overtaxing the finite resources of captive breeding programs. Ultimately, amphibian conservation strategies will benefit from the development of innovative approaches to make reintroduction efforts successful.

The microbiome of amphibian skin has been a specific research focus due to the devastating impacts of the infectious disease chytridiomycosis, which is caused by lethal pathogens that colonize the epidermis (Voyles et al., 2009). The cutaneous microbiome can contribute directly to protection from invasive pathogens (Harris et al.,

2009; Kueneman et al., 2016), and indirectly through priming immune defenses (Rollins-Smith et al., 2011). Recent studies have shown that the presence and, in some cases, the proportional abundance of bacteria that are known to inhibit Bd, can influence disease outcomes for individual amphibians and for populations of species across the landscape (Jani et al., 2017). Interactions between amphibian skin microbiota and amphibian pathogens may have consequences for amphibian communities because co-occurring amphibian species in the same habitats have distinct microbiomes (Kueneman et al., 2014), and therefore may have varying susceptibility to disease. In addition, because temperature can alter how the microbiome functions, recent research has focused on understanding how thermal conditions may impact the microbiome, thereby mediating amphibian defenses against Bd (Robak and Richards-Zawacki, 2018).

To date, our understanding of the effect of captive programs on the amphibian skin microbiome is limited. While several studies suggest that captive individuals have cutaneous microbiomes that retain lower microbial diversity compared to their wild counterparts (Becker et al., 2014; Antwis et al., 2014; Bates et al., 2019), other studies have reported no significant change (Flechas et al., 2017; Hernández-Gómez et al., 2017). Overall, in published studies on 18 amphibian species, 12 species showed decreased richness and altered bacterial composition in captive amphibians, while six species showed increases in bacterial richness or no change in microbial diversity (Supplemental Table 1). However, these studies used markedly different methods for experimental design, sample collection, and statistical analysis. These inconsistencies likely contributed to the mixed outcomes, making interpretation difficult. As such, there is a need for a reevaluation and synthesis of the available data on skin-associated microbiomes between captive and wild populations. In addition, we currently lack a deeper understanding of how the microbiome may shift – both in composition and function – as animals transition from captive conditions back into the wild during reintroduction efforts.

To advance our understanding of the amphibian microbiome in captive and wild conditions, we integrated two key approaches. First, we conducted a meta-analysis to examine the effects of captivity on amphibians using a standardized sampling protocol, a re-examination of published data, and a pipeline with identical measurement rubrics across amphibian species. We explored the consequences of captivity using a hypothesis-driven framework. Our main hypotheses were that captive conditions reduce amphibian skin microbiome diversity, antifungal function, and beta dispersion (a measure of the microbial community dissimilarity among a group of samples). In addition, we asked more specific questions concerning factors that might contribute to microbiome differences in captive populations, including (1) substrate type (semi-sterile or semi-natural), (2) hatching origin (captive or wild hatched), (3) amphibian order (Anura or Caudata), (4) and host bioregion (Temperate or Tropical species). Second, we conducted a soft-release reintroduction experiment to assess how captive amphibian skin microbiota changes over time (incorporating pre-release conditioning using outdoor mesocosms). We hypothesized that the skin microbiome – and the antifungal function of the microbiome – would shift to resemble the microbiome of wild frogs more closely. We consider the possibility that rewilding the microbiome (the process of restoring the wild-type microbiome of an animal) may improve reintroduction success by restoring beneficial microbiomes prior to release.

2. Methods

2.1. Summary of the meta-analysis

For this study, we sequenced bacterial community DNA collected on swabs gathered from 302 individual amphibians. We assembled the newly sequenced data with skin microbiome data from published studies to generate a dataset that includes samples collected from 578 individual post-metamorphic amphibians, representing 18 species and ten

amphibian families found in temperate and tropical regions. A summary of all amphibian samples included in the meta-analysis is provided in Table 1.

2.2. Skin sampling and bacterial community sequencing

We collected amphibian skin microbiota using sterile swabs and standardized sterile sampling techniques as described in (Culp et al., 2007). We extracted DNA using Qiagen Power Soil kit and amplified the V4 region of the 16S rRNA gene with barcoded primers (515f-806r) (except for one published study which sequences V2) (Hernández-Gómez et al., 2017). We sequenced samples on Illumina MiSeq and HiSeq platforms (Table 1). We compiled the raw sequence data from published studies and data from manuscripts in preparation (Table 1).

Table 1

Shown are the amphibian species sampled, numbers of samples included by captive and wild conditions, the gene region sequenced, and the citation for the data included in this study. All data were collected on an Illumina MiSeq platform except for the two studies of captive and wild *Anaxyrus boreas* collected on an Illumina HiSeq.

Species	Status	Count	Rarefaction Depth	Reference
<i>Ambystoma maculatum</i>	Captive	17	2885	Barnhart, 2018
<i>Ambystoma maculatum</i>	Wild	13	2885	This study
<i>Anaxyrus boreas</i>	Captive	9	2885	Chen et al., 2022
<i>Anaxyrus boreas</i>	Wild	19	2885	Kueneman et al., 2016
<i>Andrias japonicus</i>	Captive	21	1254	Bletz et al., 2017
<i>Andrias japonicus</i>	Wild	13	1254	Bletz et al., 2017
<i>Atelopus certus</i>	Captive	5	2885	This study
<i>Atelopus certus</i>	Wild	8	2885	This study
<i>Atelopus limosus</i>	Captive	5	2885	This study
<i>Atelopus limosus</i>	Wild	28	2885	This study
<i>Atelopus varius</i>	Captive	10	2885	This study
<i>Atelopus varius</i>	Wild	28	2885	This study
<i>Atelopus zeteki</i>	Captive	10	2885	Becker et al., 2014
<i>Atelopus zeteki</i>	Wild	28	2885	Becker et al., 2014
<i>Cryptobranchius alleganiensis</i>	Captive	20	2885	Hernández-Gómez et al., 2019
<i>Cryptobranchius alleganiensis</i>	Wild	17	2885	Hernández-Gómez et al., 2019
<i>Cynops pyrrhogaster</i>	Captive	18	1432	Sabino-Pinto et al., 2016
<i>Cynops pyrrhogaster</i>	Wild	27	1432	Sabino-Pinto et al., 2016
<i>Espadarana prosoblepon</i>	Captive	4	2885	This study
<i>Espadarana prosoblepon</i>	Wild	48	2885	This study
<i>Hylomantis lemur</i>	Captive	17	2885	This study
<i>Hylomantis lemur</i>	Wild	5	2885	This study
<i>Rana catesbeianus</i>	Captive	13	2885	Chen et al., 2022 and this study
<i>Rana catesbeianus</i>	Wild	52	2885	Kueneman et al., 2019 and this study
<i>Mantella aurantiaca</i>	Captive	8	2885	This study
<i>Mantella aurantiaca</i>	Wild	6	2885	This study
<i>Osteopilus septentrionalis</i>	Captive	9	2885	Chen et al., 2022
<i>Osteopilus septentrionalis</i>	Wild	6	2885	Kueneman et al., 2019
<i>Plethodon cinereus</i>	Captive	18	2885	Loudon et al., 2014
<i>Plethodon cinereus</i>	Wild	20	2885	Loudon et al., 2014
<i>Rana luteiventris</i>	Captive	4	1234	Loudon et al., 2020
<i>Rana luteiventris</i>	Wild	10	1234	Loudon et al., 2020
<i>Rana pretiosa</i>	Captive	31	2068	This study
<i>Rana pretiosa</i>	Wild	17	2068	This study
<i>Strabomantis bufoniformis</i>	Captive	4	2885	This study
<i>Strabomantis bufoniformis</i>	Wild	10	2885	Rebollar et al., 2016

We quality filtered the sequences and further processed them using Quantitative Insights into Microbial Ecology 2 (QIIME2 (Bolyen et al., 2019)). Sub-Operational Taxonomic Units (sOTUs) were determined using deblur (Amir et al., 2017). Within this sOTU clustering, we trimmed all sequences to 90 bp to accommodate all studies. The final dataset comprised 15,816,777 reads with a mean frequency of 28,705 and a median frequency of 18,702 reads per sample.

2.3. Assessment of microbial diversity and composition

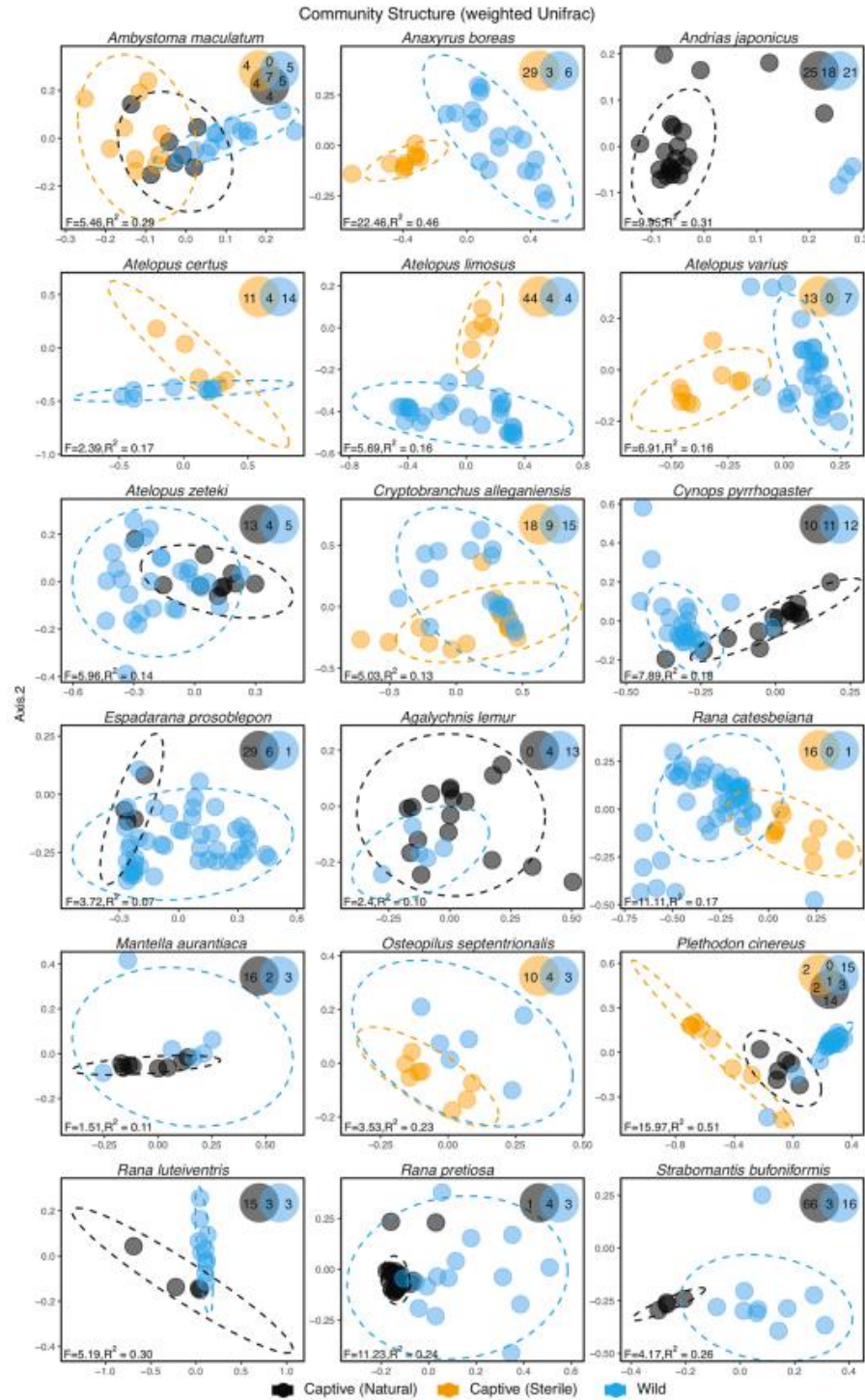
We subsequently rarefied most samples at 2885 reads per sample to allow for robust sample sizes across most species and to fully capture the microbial diversity (Supplemental Fig. 1). However, there were four species for which we rarefied at lower depths to allow for adequate sample size (Table 1). We used this approach to allow for the inclusion of amphibian species with lower sequencing depth while also providing a more in-depth analysis for species with higher sequence depth. We built a phylogenetic tree using fasttree2 using align-to-tree-mafft-fasttree, and taxonomy was assigned using sklearn and a pre-trained Greengenes database. We calculated alpha diversity [sOTU richness, phylogenetic diversity and evenness] and beta diversity (weighted and unweighted Unifrac; (Lozupone and Knight, 2005) in QIIME2. We used weighted Unifrac in our species by species (wild vs. captive) comparisons to capture the full compositional differences that exist (Fig. 1). We used unweighted Unifrac to capture the assimilation of rare taxa into the microbial community of amphibians undergoing a soft-release (Fig. 3). We subsequently analyzed and visualized the results in R (R Core Team, 2021).

2.4. Prediction of capacity to inhibit *Batrachochytrium* pathogens

We predicted *Batrachochytrium*-inhibitory function using a custom bash script (see Github: <https://github.com/m-bletz/Amphibian-Captive-Wild-Metaanalysis>) and a database containing 16S rRNA sequences from amphibian skin bacteria that have been tested for functional activity against the two *Batrachochytrium* pathogens, (*Bd*) and (*Bsal*). This database included 7382 total sequences, 1489 of which exhibited consistent inhibitory function (Woodhams et al., 2015). The bash script used vsearch to cluster sOTU sequences to potentially inhibitory sequences within the database at 99% similarity. We then calculated two response variables, antifungal function (the proportion of "inhibitory" reads with respect to the full, rarefied community) and antifungal richness (the number of inhibitory sOTUs).

2.5. Calculation of diversity and dispersion metrics

We calculated and pooled our effect sizes for three alpha diversity metrics, two functional prediction metrics, and community dispersion (specifically, the average dissimilarity from individual observation units to their group centroid in multivariate space) using the metafor package in R (Balduzzi et al., 2019; Viechtbauer, 2010). Briefly, we calculated arithmetic means, standard deviations, and sample sizes for wild and captive individuals from each species/dataset for each univariate metric. For community dispersion we calculated the average within group pairwise distances with the adegenet package in R (Jombart and Dray, 2008). We calculated bias-corrected standardized mean differences using Hedge's *g* (Hedges, 1981). We pooled and assessed these effect sizes using mixed effect models using the rma.mv() function with maximum likelihood estimation in R. We included a random term of study to account for non-independence of samples for two study species (*Plethodon cinereus* and *Ambystoma maculatum*). The rma.mv function includes a test for heterogeneity, using a generalized weighted least squares extension of Cochran's Q-test (Viechtbauer, 2010). Furthermore, we used subgroup or moderator analyses to examine a priori hypotheses about whether substrate type (semi-sterile or semi-natural), hatching origin (captive or wild hatched), amphibian order (Anura or



(caption on next page)

Fig. 1. Captivity affects skin community structure measured as between-community (beta) diversity among samples using principal coordinates analysis (PCoA) of weighted unifrac distance. Black circles represent captive individuals housed in semi-natural environments (including soil and plant substrates), yellow circles represent captive individuals housed in semi-sterile conditions (no natural substrates), and blue circles represent wild individuals collected from their natural environments. Each species comparison of beta diversity between captive and wild individuals includes a Venn diagram showing overlapping core bacteria (bacteria found in >75% of samples by type). Adonis statistics are provided in the lower left of each plot; $p = 0.001$ for all, except *Atelopus certus* = 0.04, *Cryptobranchius alleganiensis* = 0.003, *Espadarana prosoblepon* = 0.007, *Agalychnis lemur* = 0.024, *Mantella aurantiaca* = 0.124, *Osteopilus septentrionalis* = 0.008, *Rana luteiventris* = 0.003, *Strabomantis bufoniformis* = 0.003.

Caudata), and host bioregion (Tropical or Temperate) explained the observed effect sizes or reduced heterogeneity in effect sizes. We define semi-sterile substrate conditions, as a housing tank that was near empty, except for treated water, or paper towels, and semi-natural substrate conditions, as a housing tank has soil and plants. We visualized effect sizes with ggplot2 in R (Wickham et al., 2019).

2.6. Analysis of beta diversity

We examined differences in beta diversity among captive and wild individuals using `adonis()` in the `vegan` package in R for both beta diversity metrics. We produced PCoA plots in R using the `ape` package (Paradis and Schliep, 2019) to compute axes and `ggplot2` for visualization (Wickham et al., 2019). We identified differentially abundant sOTUs between captive and wild individuals for each species using linear decomposition models (LDM, (Hu and Satten, 2020)). We created heatmaps of identified differentially abundant sOTUs (q -value < 0.01) using `geom_tile()` in `ggplot2` (); see Github: <https://github.com/m-bletz/Amphibian-CaptiveWild-Metaanalysis>. In addition, we identified shared core sOTUs among captive and wild individuals within each species, as well as shared sOTUs among captive individuals across species, in R using `ps_venn()` in the `MicrEco` package (Liu et al., 2021). Core microbes are microbes that are common across individuals. We defined the core as the depth where we began to see shared taxa sOTUs for individuals of a given species. We chose 50% (core) for all species and 75% (core) for comparisons between wild and captive individuals of a given species (Fig. 1).

2.7. Rewilding of the microbiome with *Atelopus varius*

For the second part of this study, we used a species of conservation concern, the variable harlequin frog, *Atelopus varius*. This critically endangered species is bred in captivity with the aim of reintroducing and establishing viable wild populations (Lewis et al., 2019). However, like several other species, reintroduction efforts have been hampered by the persistent threat of *Bd*, which is still present and pathogenic in the native habitats of this species (Voyles et al., 2018; Linhoff et al., 2021).

Atelopus varius were captive reared from founders collected in the Donoso area of Panama. In captivity, they were maintained in same-sex groups of up to 10 individuals held in numbered glass tanks (size 25 × 53 × 38 cm) with automated misting systems lightly spraying the tank interiors with carbon-filtered water for 5 min every 2 h. Cages were initially sterilized with false bottoms installed (plastic egg crate covered in 0.5 mm screen mesh), keeping frogs out of contact with fecal pellets and any dirty water that may have pooled on the tank bottom. The false bottom was 20% covered with damp paper towel changed daily. Ultraviolet-emitting lights supplemented the 12-h overhead fluorescent lights for eight 45-min intervals per day. Each tank was furnished with one potted *Philodendron* plant. At the field site (8.91626°N 80.66267°W) located in the Donoso area, frogs were individually housed in 14 mesocosms (76 cm × 76 cm × 46 cm) built from a non-toxic, pliable, yet semi-rigid polyethylene mesh (0.6 mm) to prevent escape and exclude large predators but allow smaller invertebrates to enter the mesocosms. They were filled and kept with 2–4-in. depth of natural leaf-litter to maintain humidity and food for leaf-litter dwelling invertebrates and with a plant or piece of palm frond to allow animals to climb to elevated nocturnal sleeping positions. All the plant material used was obtained from the field site. During January 17–April 5, 2018, frogs inside the

mesocosms were monitored weekly. A skin swab for microbiome analysis was obtained on day 0 ($n = 11$ pre-release), and again on either day 27 ($n = 5$) or day 79 ($n = 7$). Frogs were rinsed with 50 mL sterile water to eliminate transient bacteria and swabbed using a sterile rayon-tipped swab (MW113, Medical Wire & Equipment). Swabs were obtained by rubbing their skin for a total of 70 strokes, i.e., 10 times on the venter, 10 times on the dorsum, 10 times on each flank, 5 times on the ventral surface of each thigh and 5 times on each palmar and plantar. Skin swabs were kept in ice during fieldwork and stored at -20°C in the laboratory. All amphibian sampling was conducted following IACUC approval and miAmbiente permitting.

3. Results

In our meta-analysis, we analyzed alpha diversity, predicted antifungal function and bacterial community composition from 20 published and unpublished studies representing 578 individual amphibians. We compared captive and wild samples for amphibian species collected from 10 families, 13 genera, and 18 species of frogs, toads, and salamanders across temperate and tropical localities (Table 1). In our field trial, we analyzed an additional 23 samples from *Atelopus varius* individuals to test for a rewilding response of skin microbiomes from captive individuals moved to outdoor enclosures.

3.1. Microbiome richness and predicted function

We calculated the effect sizes (Hedges g) for three alpha diversity metrics: richness, phylogenetic diversity and evenness (Fig. 2); two functional prediction metrics: antifungal richness, antifungal function (Fig. 2); and one beta diversity metric: community dispersion (Fig. 2). The overall effect of captivity (measured by Hedge's g) for richness and phylogenetic diversity were significant ($p = 0.02$ and $p = 0.04$, respectively) and predicted antifungal function approached significance ($p = 0.08$). We observed a lower richness and phylogenetic diversity in captive species for 13 out of 20 captive/wild comparisons, and a higher antifungal function in 12 out of 20 captive/wild comparisons. However, for evenness and antifungal richness, we did not observe a significant overarching effect of captivity (Fig. 2), and there was high heterogeneity among studies (Supplemental Table 2). We used subgroup analyses to explore the observed effect sizes and whether our four a priori hypotheses explained the observed variation (see methods). In all cases, the magnitude and direction of effect sizes did not support our hypotheses, and differences were non-significant, concerning substrate type (semi-sterile or semi-natural), hatching origin (captive versus wild hatching), amphibian order (Anura vs. Caudata), and host bioregion (Tropic vs. Temperate) (Supplemental Table 3).

3.2. Microbiome dispersion

In contrast, there was a significant overarching effect of captivity on community dispersion (SMD = -1.07 , $p < 0.001$; Fig. 2). In nearly all cases (19/20), there was lower community dispersion for captive individuals compared to wild individuals. None of the moderators associated with our a priori hypotheses were significant ($p \geq 0.05$).

3.3. Microbiome composition

Except for one species (*Mantella aurantiaca*), we found significant

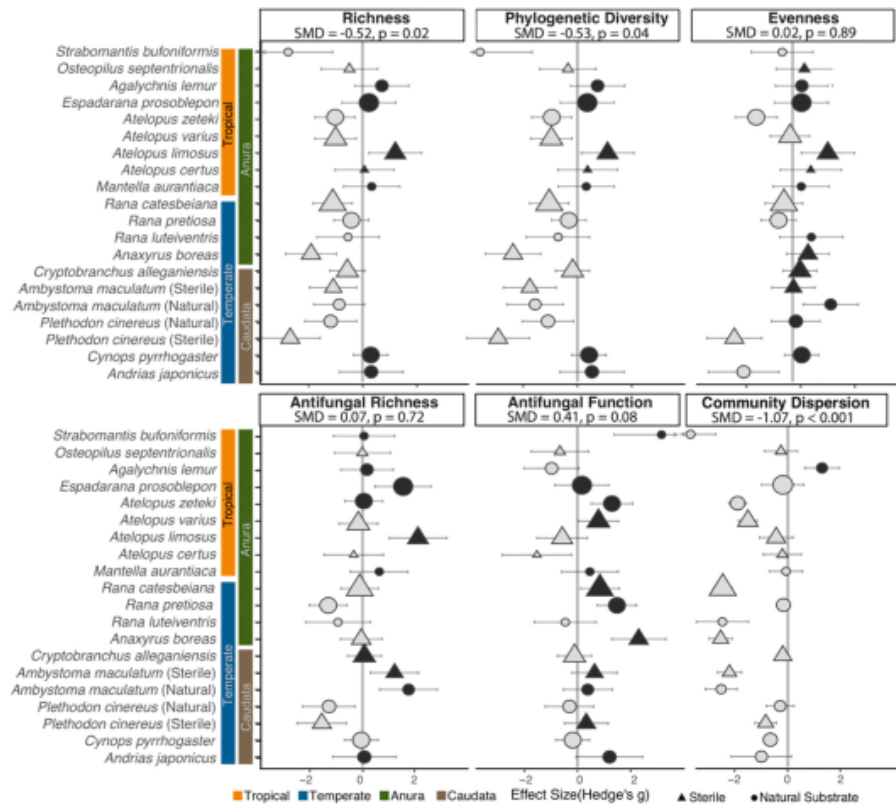


Fig. 2. Magnitude and direction of effect sizes vary across the studied species. Six panels show the mean effect sizes (Hedge's g, bias corrected standardized mean difference) across 18 studied species for alpha diversity (richness, phylogenetic diversity and evenness), beta diversity (dispersion) and antifungal prediction metrics (antifungal richness, antifungal function). Negative values indicate a reduction due to captivity, whereas positive values indicate an increase associated with captivity. Point size is scaled by sample size, and shape denotes substrate type (natural vs sterile) in captivity. Overall standard mean difference (SMD) and random effect model p-values are provided for each metric. Vertical color bars indicate amphibian order (Anura, Caudata) and bioregion (temperate, tropical).

differences in amphibian skin microbial community structure between captive and wild groups (Fig. 1). Adonis $p < 0.001$ for most species, and Adonis p values *Atelopus certus* = 0.04, *Cryptobranchus alleganiensis* = 0.003, *Espadarana prosoblepon* = 0.007, *Agalychnis lemur* = 0.024, *Osteopilus septentrionalis* = 0.008, *Rana luteiventris* = 0.003, *Strabomantis bufoniformis* = 0.003 (Fig. 1). Despite being significantly different in nearly all cases, the degree of community similarity between captive and wild individuals of the same species differed by species. Skin bacteria of captive individuals of *Anaxyrus boreas*, *Andrias japonicus*, *Atelopus limosus*, and *Plethodon cinereus* fell outside the community space of wild individuals (weighted Unifrac, visualized by the 95% confidence ellipses), whereas skin bacteria of captive individuals of *Mantella aurantiaca* and *Rana pretiosa* fell inside the community space of wild individuals. Indeed, the most common outcome was minimal to moderate overlap of community space between captive and wild individuals of the same species (Fig. 1).

To explore compositional differences of amphibian skin microbiomes in distinct habitat types (wild vs. captive) in more detail, we considered the conditions of captivity for each species. For instance, whether the individuals within a species were housed on semi natural substrates or semi-sterile substrates. Indeed, substrate type appeared to influence the differences between captive and wild individuals of an amphibian species. For the few species ($N = 2$) in which we have individuals housed in

both semi-natural substrate conditions and semi-sterile substrate conditions (*Ambystoma maculatum*, *Plethodon cinereus*), we detected greater differences in bacterial community beta dispersion between captive and wild groups for individuals housed in semi-sterile conditions (Fig. 2).

3.4. Core microbiomes

We found no bacterial sOTUs that were shared across all wild amphibians, no taxa that were shared among all captive individuals on semi-sterile substrates, and only two bacterial sOTUs that were shared by captive individuals housed with semi-natural substrates. Two bacterial taxa were shared between wild and captive amphibians housed with semi-natural substrates, and two bacterial taxa were shared between captive amphibians housed with semi-sterile substrates and semi-natural substrates. When we consider overlapping core bacteria between captive and wild individuals of the same species (bacteria found in >75% of samples by type), we found that all amphibians except *Atelopus varius* and *Rana catesbeiana* shared at least 1 core sOTU (Fig. 1, Supplemental Github; <https://github.com/m-bletz/Amphibian-CaptiveWild-Metaanalysis>).

3.5. Rewilding of the microbiome

One species within our study, *Atelopus varius*, was raised in captivity and then transferred to mesocosms and repeatedly sampled through time. This soft-release provided an opportunity to see how microbiomes shift when captive amphibians are returned to more semi-natural substrates. When amphibians were placed in mesocosms their skin microbiomes shifted to become more like the wild type over time. The PCOA unweighted Unifrac analysis revealed that individual samples at later time points in the mesocosms are more like wild sampled individuals (Adonis: Pseudo-F = 9.8521, $R^2 = 0.13905$, $p = 0.001$ and all pairwise comparison are significant $p = 0.01$, except mesocosms day 27, and day 79 where $p \geq 0.05$) (Fig. 3). We found that the percent of the bacterial community that matched to sequences with predicted antifungal function increased through time spent in the mesocosms (median value $\pm$ SE: mesocosm time point 0 = 0.39 ± 0.03 , mesocosm time point 27 = 0.53 ± 0.08 , and mesocosm time point 79 = 0.61 ± 0.09). Inferred antifungal function of the microbiome also increased through time spent in mesocosms (correlation tau = 0.43, $z = 2.56$, p -value = 0.01).

4. Discussion

The reliance on captive breeding programs has greatly increased in recent decades and amphibians have experienced the greatest numbers of species moved into captivity relative to any other class of vertebrates (Conservation Needs Assessment, 2019). We investigated the consequences of captivity on the skin microbiome of a diverse group of amphibians by synthesizing published data, generating additional data from new samples, and conducting a meta-analysis to probe specific questions concerning the microbiome in captive amphibians. We also conducted a soft-release reintroduction experiment to determine if the captive amphibian skin microbiota could shift over time to resemble more closely that of wild amphibians. Here, we provide a novel

assessment that was only possible with assistance from amphibian conservation research communities, consistent methodological efforts, and standardized practices for animal handling, sampling, DNA extractions and sequencing (Thompson et al., 2017; Kueneman et al., 2019).

Although it is generally assumed that animals experience a loss of microbial diversity when they are moved into captivity (Kohl et al., 2014; Clayton et al., 2016; Borbón-García et al., 2017) and that amphibians lose protective skin microbiota, the first part of our study suggests that this assumption is not necessarily true. While our meta-analysis revealed an overarching responses of a decrease in amphibian skin microbial diversity in captivity, as measured by richness and phylogenetic diversity. We also observe an increase in amphibian skin microbial diversity for 35% (7/20) of the species in our study.

This finding may be explained by the fact that we detected high heterogeneity among studies, suggesting strong differences in the skin ecology of different amphibian species and/or that differences in experimental design that may account for inconsistencies in the direction of the shift in microbial diversity among amphibian species. The predicted antifungal function of the microbiome across all studies was only modestly higher in captive amphibians compared to their wild counterparts. While we did not directly test antifungal function (we made an inference from a database of microbial function), this finding of near significant ($P = 0.08$) increases in antifungal function in captivity is intriguing and worth further investigation. The presence of microbes with antifungal function may decrease infection risk and intensity (Kueneman et al., 2016; Chen et al., 2022). Irrespective of microbial function, our finding that some amphibians exhibited higher microbial richness when in captivity challenges the previously held notion that captivity necessarily leads to depletion of the microbiome (Bates et al., 2019; Chong et al., 2019).

We found that the community composition of amphibian skin microbiomes is consistently different between captive held individuals

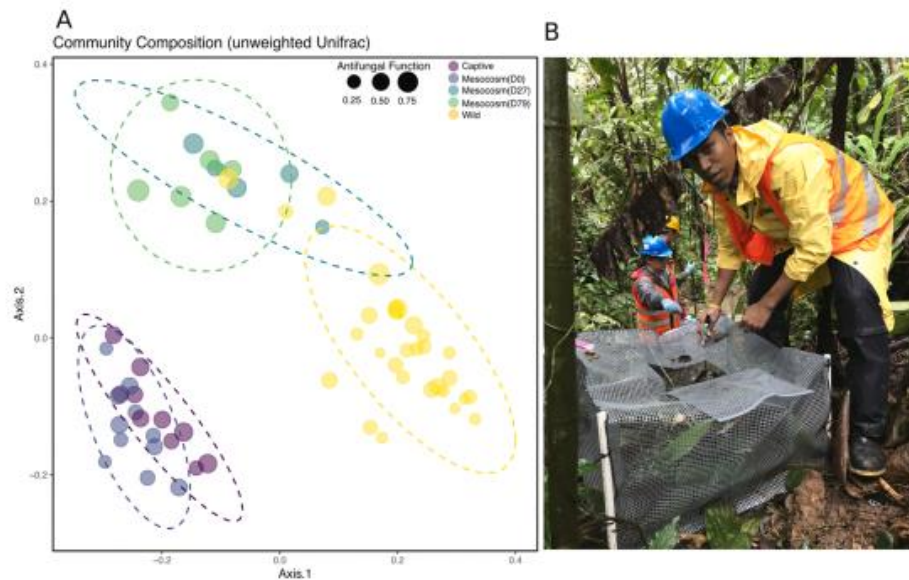


Fig. 3. Community composition *Atelopus varius*. (A) Community composition of *Atelopus varius* transitioning to wild habitats. Unweighted unifrac distances of juvenile *A. varius* born in captivity, transitioned to outdoor mesocosms (D0, D27, D79) and wild-caught field individuals. Sample groups are significantly distinct from one another Adonis: Pseudo-F = 9.8521, $R^2 = 0.13905$, $p = 0.001$ and all pairwise comparisons are significant $p = 0.01$, except mesocosms day 27, and day 79 where $p \geq 0.05$). Later mesocosm timepoints are closer to wild caught individuals from native habitats. Antifungal function (the proportion of the microbial community that matches known *Bd* inhibitory isolates) is depicted by the size of the spheres. (B) Image of *A. varius* in mesocosms.

and their wild counterparts (Species $N = 19/20$). These community differences could be a result of an increase or decrease of the abundance of certain microbial members, which could lead to differences in the overall composition of its microbiome. An additional component of skin microbial composition that could evoke differences between captive and wild group of samples is their dispersion. We measured this component of beta diversity to address our hypothesis that captive amphibians generally display greater microbiome homogeneity, while wild amphibians may exhibit greater microbiome dispersion. We found a significant effect of captivity on skin microbial homogeneity. Within amphibian species, captive animals had greater homogeneity, i.e., more similar microbiome composition to each other in comparison to wild animals that had more variability in the microbiomes. This result was expected, as amphibians in captivity are believed to be deprived of the same degree of microbial diversity that individuals of the same species are exposed to in the wild. Intriguingly, the one species (*Agalychnis lemur*) that had greater microbial community variation also had greater richness in captivity. This finding suggests that amphibian behavior or some other species-specific variable may play a role in decreasing skin microbial diversity in natural conditions. Indeed, a better understanding of the links between amphibian behavior and the microbial community may advance our understanding of the links between the microbiome and amphibian health in both captive and wild settings.

Using our meta-analysis we addressed additional specific questions concerning factors that might contribute to microbiome differences in captive populations, including the use of artificial housing substrates, captive hatching of the animals versus wild hatching, regional point of origin (e.g., temperate and tropical species), and amphibian order (e.g., Anura versus Caudata). To begin with, we detected greater, but non-significant, bacterial community differences between captive and wild groups for individuals housed with semi-sterile substrates compared to semi-natural substrates. As such, the substrate type that is used in captivity could potentially change the directionality of the richness response to captivity or further reinforce the captivity effect for a given species. Moreover, we compared the origins of amphibians in captivity by comparing captivity effects for amphibians raised in captivity to those that were brought into captivity and had been there for several months. We did not find a significant difference between these groups, suggesting that the effects of captivity on the skin-microbiome can occur rather rapidly, as demonstrated by Loudon et al. (2014). Additionally, we explored the hypotheses that host bioregion and host order may be important determinants of the effect of captivity on microbiome diversity and function. We did not find either factor (bioregion or host order) to have significant effects on the response of skin microbiome richness, community dispersion, or anti-fungal function to captivity. Instead, we found the strongest effects at the species level. Even within the same genera, amphibian species can behave uniquely with respect to their skin microbiome's response to captivity. Taken together, we report that captive conditions influence the composition of amphibian skin microbiome in predictable ways (altered composition, and decreased dispersion), but skin-microbial alpha diversity, antifungal richness and antifungal function have more variable responses than we had predicted.

In the second part of this study, we found that moving captive amphibians to experimental soft-release mesocosms (pre-release conditioning) resulted in rapid shifts in microbiome diversity, which we refer to as a rewilding the microbiome. While it is generally understood that the microbiome can shift over prolonged time scales (e.g., over the epizootic to enzootic transition (Jani et al., 2017), and over seasons (Longo et al., 2015; Tong et al., 2020; Le Sage et al., 2021), our results indicate that the microbiome shifted over a short amount of time for individuals that were moved out of a captive breeding program. Specifically, we found that a soft-release of captive-bred *A. varius* into outdoor mesocosms with natural substrates and food sources caused a shift in the skin microbiome toward a more wild-type microbial composition within 27 days. Furthermore, we estimated that the anti-

Batrachochytrium function of the microbiome increased in mesocosms during this study. These findings provide further evidence that the microbiome is a naturally occurring mechanism helping to confer amphibian resistance to fungi such as *B. dendrobatidis* infection and they suggest that rewilding the microbiome can be an effective way to increase the success of reintroduction programs for some species.

4.1. Conservation implications

Conservation groups and agencies are often put into the position of either losing an amphibian species entirely or safeguarding the remaining depleted population by establishing ex situ programs and breeding them in captivity until conditions in the wild improve enough to reintroduce these threatened species (Linhoff et al., 2021). Safeguarding amphibians introduces substantial logistical challenges of how to care for a species and whether its new environment is sufficient for a species to develop and to reproduce. Supporting beneficial microbiomes in captivity is one tool conservation managers could use to improve the outcome of breeding and reintroduction programs. Comparing the skin microbiome from natural amphibian populations offers a vision of what the skin microbiomes of a captive-raised individual should re-wild into. Rewilding may be enhanced by incorporating wild individuals or natural substrates into the re-wilding protocol, thus facilitating acquisition of naturally occurring microbes. However, this approach should be weighed against the risk posed to the wild amphibians involved, and the risk of parasite transmission into captive colonies.

The composition and function of the microbiome may be one of several mechanisms that have facilitated disease resilience and allowed species to persist, and even recover from initial chytridiomycosis outbreaks (Voyles et al., 2018). Our results suggest that using prerelease conditioning, such as innovative soft-release approaches to "rewild the microbiome", may improve the likelihood of survival in reintroduction programs. In doing so, we found that *A. varius* can be rehabilitated to a more natural wild-type skin microbiome with higher anti-*B. dendrobatidis* function through the course of our study, increasing through time spent in the outdoor mesocosms. Indeed, unpublished work also found that a soft-release improved survivorship of frogs, even though the odds of becoming infected also rose from the extra time exposed to *B. dendrobatidis* in the field (Estrada et al. in review). If native microbial defenses do not facilitate survival in landscapes with severe risk from skin pathogens, and rewilding is insufficient to support recovery of the species, more imaginative tools may be needed. Such tools could include the introduction of novel, altered, or even genetically modified microbiomes. In many cases the efficacy of such approaches may be short term, as the return of animals to wild-like conditions appears to facilitate the re-establishment of naturally occurring microbial associations. Given the severity of the amphibian decline crisis, more advanced strategies in conservation management are needed, but can also raise substantial ethical issues, or carry risks that should be fully considered.

The results of this meta-analysis and soft-release for the goal of rewilding the microbiome can broadly inform strategies of captive breeding and reintroduction. The direction and the magnitude of the effect of captivity is not predetermined, and this study dispels conventional wisdom suggesting captivity always decreases microbial diversity, and protective components of the microbiome. Natural substrate conditions can help maintain wild-type microbial communities, and while microbial variability will likely be reduced in captivity, a soft-release can help recover beneficial groups of protective microbes if they are lost in captivity. Thus, we must continue to use science-based approaches to improve reintroduction efforts for the good of all species that require ex situ captive programs due to a variety of threats, and we must learn what we can from the successes and failures of all captive breeding programs.

Statement

We confirm that all the reported work is original, that this manuscript has not been submitted for publication elsewhere, and we have no conflicts of interest. Furthermore, all authors have seen and approved the final version submitted, all prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocon.2022.109576>.

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